The STAT3-VDAC1 axis modulates mitochondrial function and plays a critical role in the survival of acute myeloid leukemia cells

Kellen B. Gil,[1] Jamie Borg,[2] Rosana Moreira Pereira,[3] Anagha Inguva-Sheth,[3] Geovana Araujo,[3] Jeremy Rahkola,[4] William Showers,[3] Abby Grier,[5] Angelo D'Alessandro,[6] Clayton Smith,[3] Christine McMahon,[3] Daniel A. Pollyea,[3] Austin E. Gillen[3,4] and Maria L. Amaya[3,4]

[1]Department of Internal Medicine, University of Colorado School of Medicine, Aurora, CO; [2]Denver Research Institute, Aurora, CO; [3]Division of Hematology, University of Colorado Anschutz Medical Campus, Aurora, CO; [4]Research Department, Rocky Mountain Regional VA Medical Center, Aurora, CO; [5]Department of Medicine, Case Western University, Cleveland, OH and [6]Department of Biochemistry and Molecular Genetics, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

Correspondence: M.L. Amaya
maria.l.amaya@cuanschutz.edu

Received: January 10, 2025.
Accepted: June 6, 2025.
Early view: June 19, 2025.

https://doi.org/10.3324/haematol.2025.287352

SUPPLEMENTAL MATERIALS

ADDITIONAL METHODS

Cell Culture

For human primary AML samples, base media of minimum essential medium (StemCell Technologies 09655) and 5.5 mM of glucose was used, supplemented with physiologic levels of amino acids and 10 nM of human cytokines stem cell factor (SCF) (Peprotech 300-07-100), interleukin-3 (Peprotech 200-03-50), and Fms Related Receptor Tyrosine Kinase 3 (FLT3) (Peprotech 300-19-100). The MOLM-13 and THP-1 cell lines were used, which was purchased from the University of Colorado Cell Technologies Shared Resource (CTSR), which was obtained from DSMZ.

Viability Measured by Flow Cytometry

Cell viability was measured using Annexin V (BD 556421) and Dapi (BD 564907) stains and measured by flow cytometry. Ghost dye (Tonbo Bioscience 130865T100) was also used to confirm cell death and measured by flow cytometry.

Mouse Studies

In vivo

NSG-SGM3 mice (Jackson Laboratory 013062) were conditioned with 25 mg/kg busulfan (Alfa Aesar J61348) via intraperitoneal injection. The following day, human primary AML cells were washed with FACS buffer and resuspended in saline with the addition of anti-human CD3 antibody (OKT3 BioXCell BE0001-2) at a final concentration of 1 μg/10^6 cells and incubated for 15 minutes prior to injection as a means to reduce graft versus host disease. 8-9 mice per group were injected with 1 x 10^6 cells/mouse. Once engraftment was confirmed on a sacrificed mouse, mice were subsequently treated with either daily IP injection 7 mg/kg Stattic or vehicle control for 6 days, followed by sacrifice. After sacrifice, femurs were collected and flushed with FACS buffer. Flow cytometry was then performed after staining

with mouse (BD 560510) and human (BD 561865) specific CD45 antibodies, human CD34 (BD 562383) and human CD38 (BD 356616) antibodies. All animal experiments were approved by Rocky Mountain Regional VA Medical Center under IRB protocol #CD2114M.

Single Cell RNA Sequencing

Clusters were annotated using clustifyr 1.9.1 and the leukemic/normal bone marrow reference dataset generated by Triana and colleagues. Scanpy and Seurat 4.1.1 were then used to generate uniform manifold approximation and projections from the TotalVI embeddings and perform exploratory analysis, and data visualization.

siRNA Transfections

siRNA sequences targeting STAT3 (siSTAT3) and a scrambled control (siSCR) were purchased directly from Horizon Discovery's ON-TARGETplus siRNA Reagents collection (L-003544-00-0005 (Stat3) and D-001810-10-05 (SCR)). The lyophilized siRNA products were resuspended in RNAse-free water at 5 μmol/L, which was used as a stock solution. $2x10^6$ cells were suspended in 80 μL of Buffer T, and 20μL of siRNA stock solution was added. These cells were then electroporated using the Neon Electroporation Transfection System (Thermo) according to the manufacturer's protocol using the following settings: 1,600 V, 10 ms, 3 pulses.

Mitochondrial Oxygen Flux Analysis

Oxygen consumption rate (OCR) was measured using the Seahorse XF96 Cell Mito Stress Test kit (Agilent 103015-100) on the Seahorse XFe96 Extracellular Flux Analyzer (Agilent) according to manufacturer's protocol. Treated MOLM-13 cells were washed and plated on Cell-Tak (Corning 354240) coated XFe96 cell culture microplates (Agilent) at 150,000 cells/well in 5 replicates using Seahorse Assay RPMI Medium (Agilent). OCR was measured at basal level and after injection of 5 μg/mL oligomycin, 2 μM Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), 5 μM antimycin A, and 5 μM rotenone.

Western Blots

Cells were lysed in RIPA Buffer (Sigma-Aldrich R0278) supplemented with HALT Protease and Phosphatase Inhibitor Cocktail (Thermo 78442). Proteins were separated by SDS-PAGE gel (Bio-Rad 4561094), transferred to PVDF membrane (Millipore 03010040001), and blocked for 1 hour at room temperature using 5% w/v BSA in 1% Tween-20-TBS Buffer. Membranes were incubated overnight at 4°C with primary antibody against pStat3 Ser727 (CellSignaling 9134S), Stat 3 (Cell Signaling 12640S), VDAC1/Porin (Abcam ab14734), CoxIV (CellSignaling 4850S) or β-Actin (SantaCruz Biotechnology SC-47778). Membranes were washed and incubated with 1:10,000 secondary antibody against mouse (Abcam ab205719) or rabbit (Abcam ab205718) for 1 hour at room temperature. The band were visualized using LumiGlo Chemiluminescent Substrate System (SeraCare 5430-0040) and the ChemiDoc MP Imaging System (Bio-Rad).

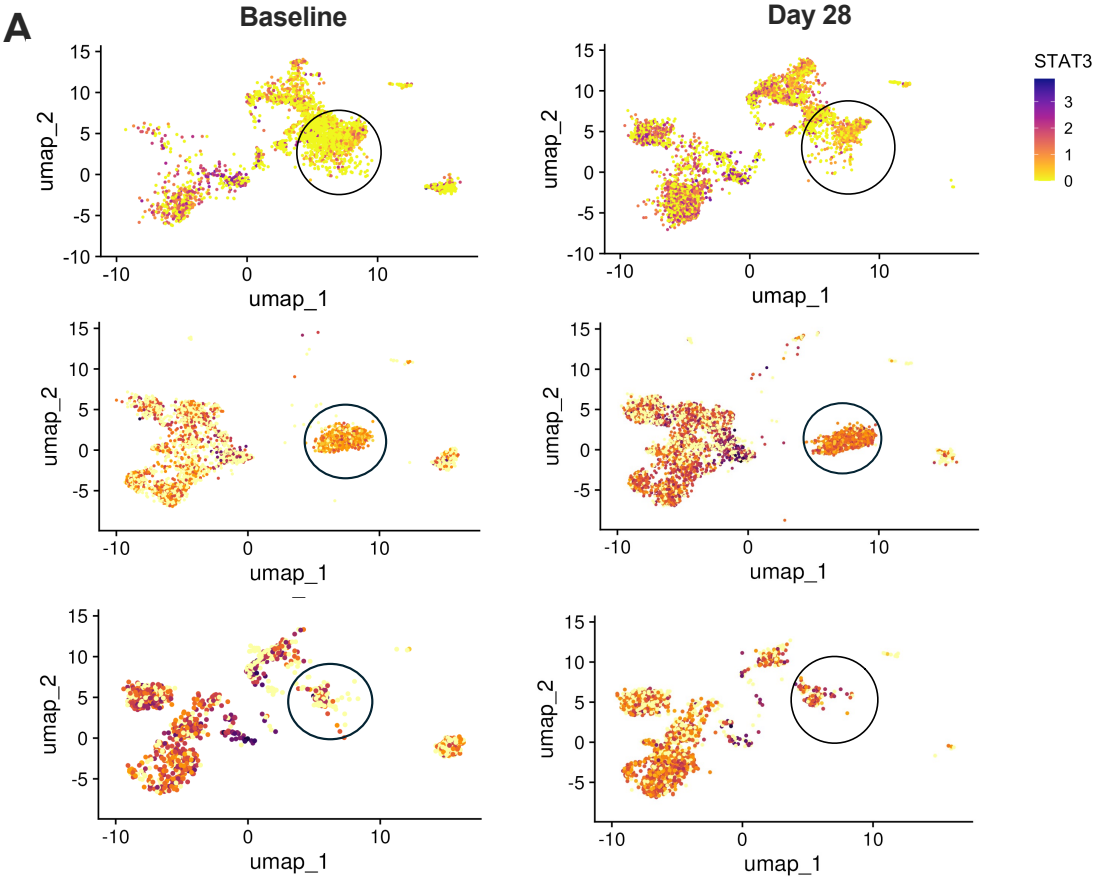
Metabolomic Experiments

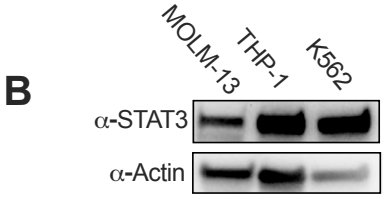
Metabolomics analyses were performed on 10 μl of sample extracts via ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry (Vanquish – QExactive – Thermo Fisher, San Jose, CA, USA) using a high-throughput 5 min gradient-based method.

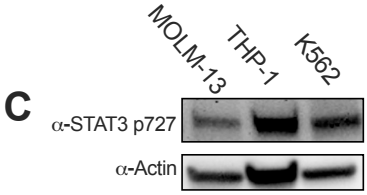
Primary Patient Samples Characteristics

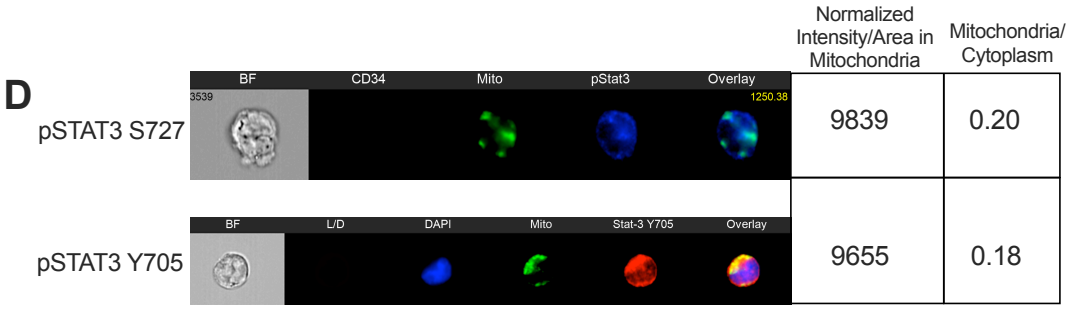
Diagnosis	Cytogenetics/FISH	Mutations	Experiment	Notes
Relapsed AML	46,XX,add(14)(q22)[4], 46,XX[16]	FLT3, NPM1 and IDH1	-In vitro viability (Figures 5C). -PDX (Figure 5D)	Venetoclax resistant
AML	46,XX,t(6;11)(q27;q23)[18]/92,slx2[2]/ KMT2Ar	KRAS, PTPN11	-PDX (Figure 5D) -In vivo study (Suppl Figure 5B)	
AML	Complex karyotype	CBL, SRSF2, TET2	-PDX (Figure 5D)	
AML	46,XX[20]	NPM1, TET2, PTPN11, ASXL1, BCOR, DNMT3A, NOTCH1, CBL FLT-TKD	-In vitro viability (Figure 5C)	Venetoclax resistant
AML	Complex karyotype/KMT2Ar	KRAS, PTPN11	-In vitro viability (Figure 5C)	Venetoclax resistant

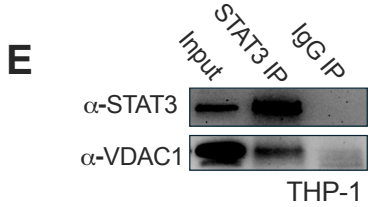
SUPPLEMENTAL FIGURE 1





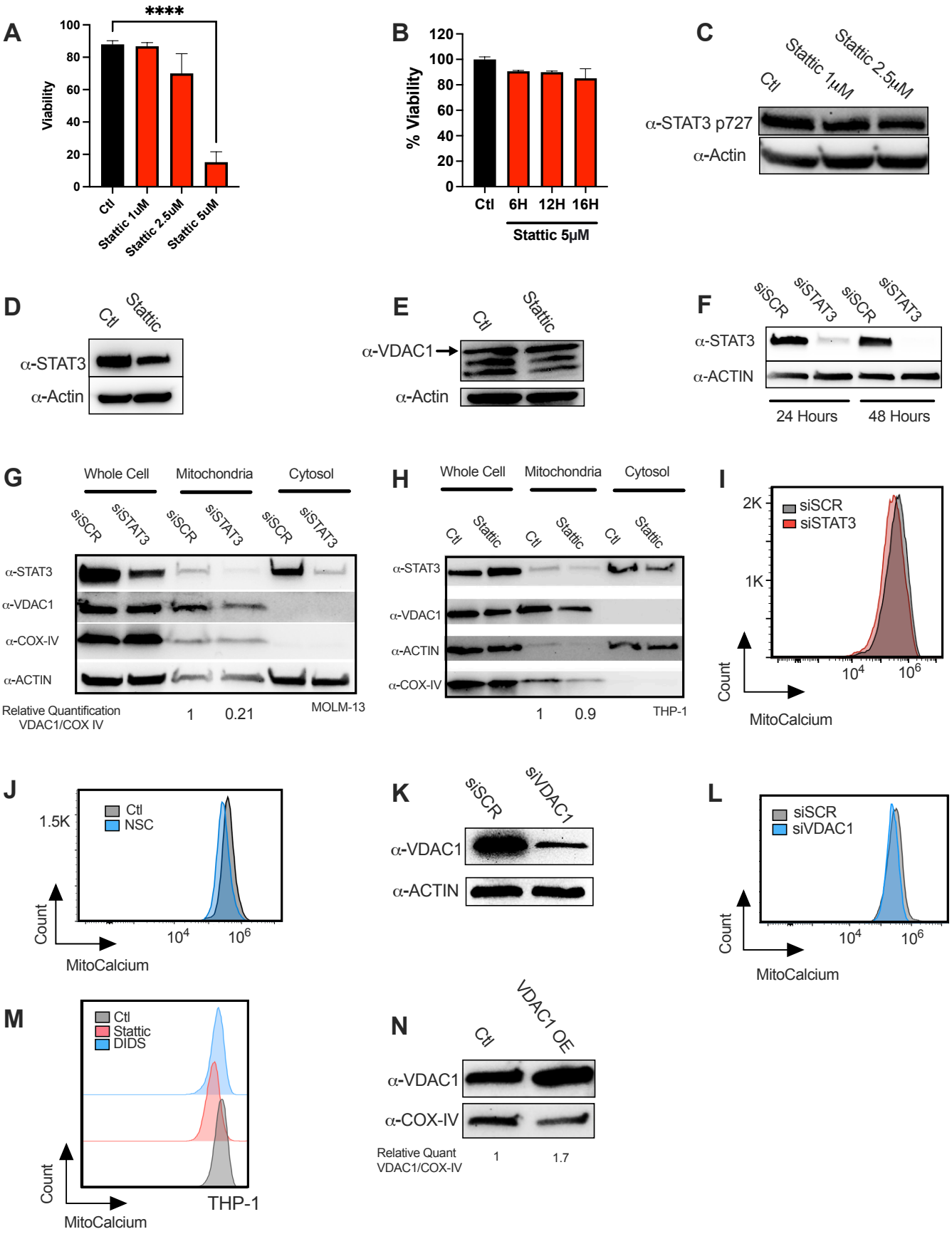






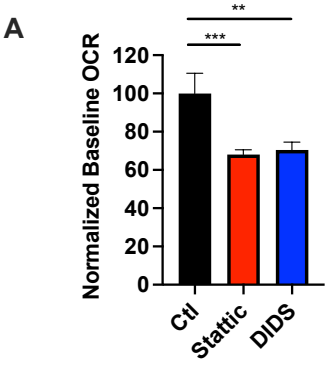
Supplemental Figure 1. **STAT3 is expressed in AML samples resistant to venetoclax and it localizes to the mitochondria of AML cells.** (A) STAT3 expression based on single cell RNA sequencing from 3 AML samples. Circled cluster shows leukemia stem cells. (B) Western blot showing STAT3 expression in MOLM-13, THP-1 and K562 cells. (C) Western blot showing phosphorylated STAT3 expression at the S727 site in MOLM-13, THP-1 and K562 cells. (D) ImageStream from cells showing STAT3 localization to the mitochondria in MOLM-13 cells based on their phosphorylation status (phosphorylated STAT3 at S727 or Y705). Intensity/area measured in all cells show marginally higher localization of pSTAT3 S727 compared to pSTAT3 Y705. (E) Western blots from STAT3 pulldown in mitochondrial extracts showing STAT3 and VDAC1 protein expression in THP-1 cells.

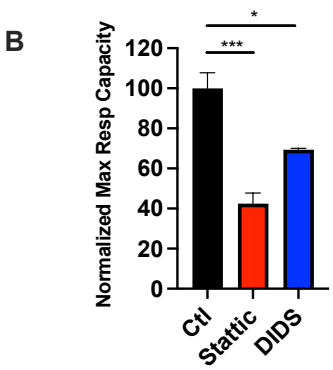
SUPPLEMENTAL FIGURE 2

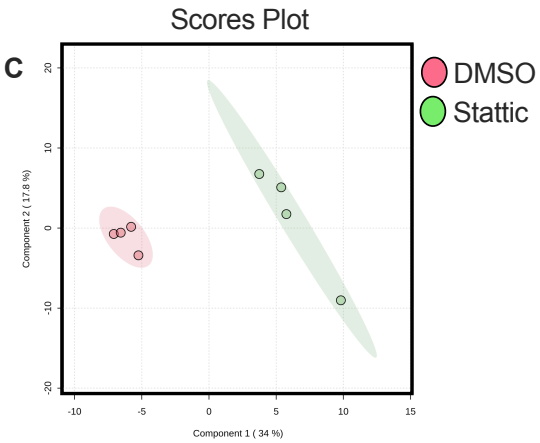


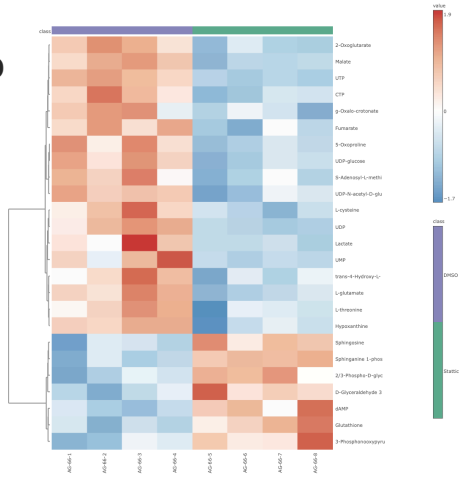
Supplemental Figure 2. STAT3 regulates mitochondrial VDAC1 and affects calcium metabolism. (A) Viability as measured by flow cytometry of MOLM-13 cells treated with increasing doses of Stattic for 24 hours. (B) Viability as measured by flow cytometry of MOLM-13 cells treated with Stattic 5μM at various timepoints. (C) Western blot showing phosphorylation of STAT3 at S727 in MOLM-13 cells after culture with 1μM and 2.5μM of Stattic for 9 hours. (D) STAT3 protein expression by western blot in MOLM-13 cells after treatment with Stattic 5μM for 9 hours or vehicle control. (E) VDAC1 protein expression by western blot in MOLM-13 cells treated with Stattic 5μM for 9 hours or vehicle control. (F) STAT3 protein expression by western blot in MOLM-13 cells treated siRNA against STAT3 or scrambled control for 24 and 48 hours. (G) Western blot showing protein expression of STAT3 and VDAC1 in whole cell, mitochondrial or cytosolic fractions of MOLM-13 cells treated with siRNA against STAT3 or scrambled control for 48 hours. COX-IV and Actin antibodies serve as mitochondrial and cytosolic controls, respectively. VDAC1/COX-IV quantification from western blot is shown below the figure. (H) Western blot showing protein expression of STAT3 and VDAC1 in whole cell, mitochondrial or cytosolic fractions of THP-1 cells treated with DMSO control or Stattic for 9 hours. COX-IV and Actin antibodies serve as mitochondrial and cytosolic controls, respectively. VDAC1/COX-IV quantification from western blot is shown below the figure. (I) Mitochondrial calcium as measured by flow cytometry of MOLM-13 cells treated with siSCR or siSTAT3 for 48 hours. (J) Mitochondrial calcium as measured by flow cytometry of MOLM-13 cells treated with the VDAC1 inhibitor NSC or vehicle control for 9 hours. (K) Western blot showing protein expression of VDAC1 in MOLM-13 cells treated with siRNA against VDAC1 or scrambled control for 48 hours. (L) Mitochondrial calcium as measured by flow cytometry of MOLM-13 cells treated with siSCR or siVDAC1 for 48 hours. (M) Mitochondrial calcium as measured by flow cytometry of THP-1 cells treated with DMSO control, 5μM Stattic or 400μM DIDS for 9 hours. (N) Western blot showing protein expression of VDAC1 in mitochondrial fraction of MOLM-13 cells treated with VDAC1 overexpression vector or vector control. COX-IV loading control expression was used for normalization.

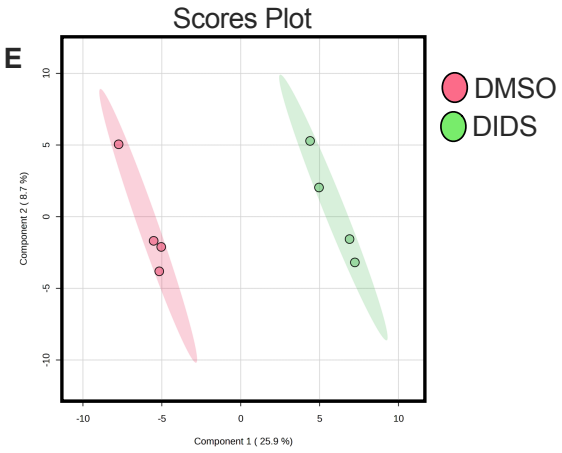
SUPPLEMENTAL FIGURE 3

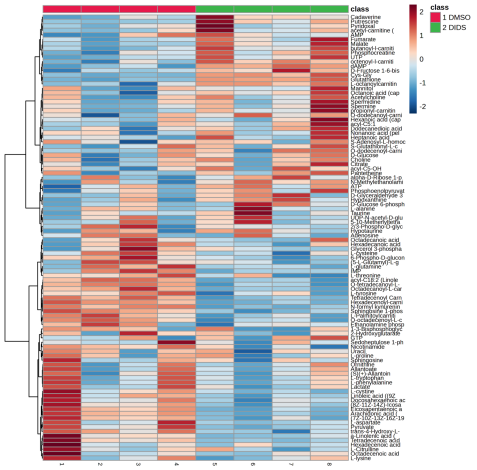


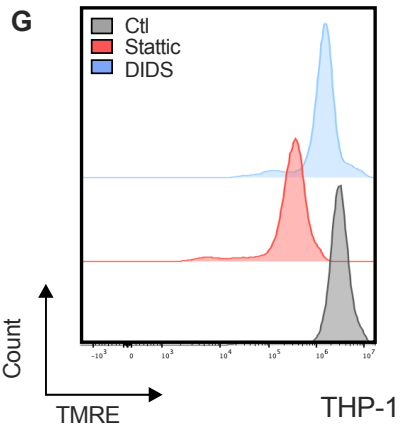






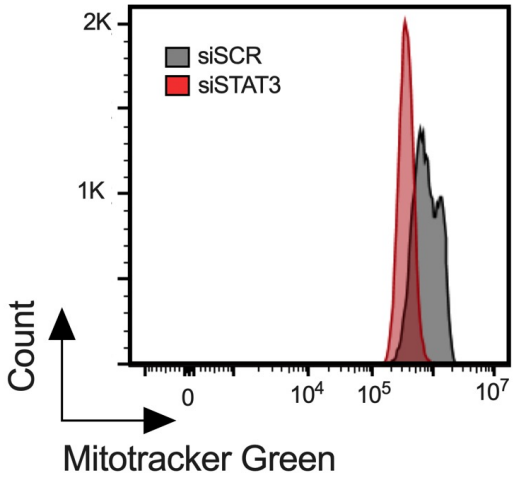






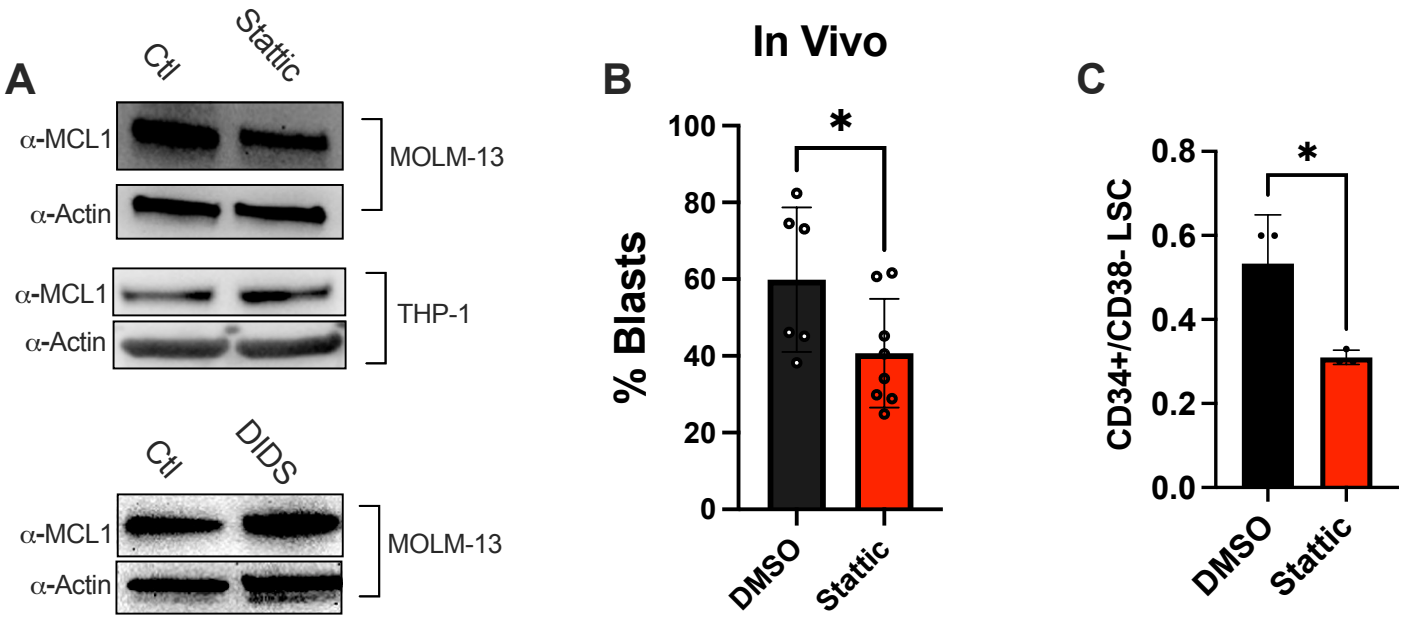
Supplemental Figure 3. STAT3 and VDAC1 inhibition results in altered metabolic properties in AML cells. (A) Quantification of Seahorse Mito Sress Test at baseline oxygen consumption rates (OCR) of THP-1 cells treated with 5μM Stattic, 400μM DIDS or vehicle control for 9 hours. (B) Quantification of Seahorse Mito Sress Test at maximal respiratory capacity of THP-1 cells treated with 5μM Stattic, 400μM DIDS or vehicle control for 9 hours. (C) Scores plot from global metabolomics of MOLM-13 cells treated with 9 hours of Stattic 5μM or vehicle control. (D) Heatmap showing altered metabolites of MOLM-13 cells treated with 9 hours of Stattic 5μM or vehicle control. (E) Scores plot from global metabolomics of MOLM-13 cells treated with 9 hours of the VDAC1 inhibitor DIDS 400μM or vehicle control. (F) Heatmap showing altered metabolites of MOLM-13 cells treated with 9 hours of DIDS 400μM or vehicle control. (G) Mitochondrial membrane potential as measured by TMRE stain of THP-1 whole cells treated with Stattic 5μM or DIDS 400μM for 9 hours compared to vehicle control.

SUPPLEMENTAL FIGURE 4



Supplemental Figure 4. **STAT3 inhibition leads to decreased mitochondrial mass.** Flow cytometry showing mitotracker green stain of MOLM-13 cells treated with siSCR or siSTAT3 for 48 hours.

SUPPLEMENTAL FIGURE 5



Supplemental Figure 5. **STAT3 Inhibition leads to AML cell death**. (A) Western blot showing MCL-1 protein levels in MOLM-13 and THP-1 cells after culture with 5μM Stattic or vehicle control for 9 hours, and in MOLM-13 cells after culture with 400μM DIDS or vehicle control for 9 hours. (B) Leukemia burden as measured by human CD45 positive AML cells of PDX mice treated with 7mg/kg Stattic or vehicle control for 7 days. (C) Leukemia stem cell burden, as measured by human CD34+/CD38- cells, of PDX mice treated with 7mg/kg Stattic or vehicle control for 6 days. Leukemia cells were pooled from each mouse group from experiment in (B), and flow cytometry was used to quantify the leukemia stem cell population (CD34+/CD38-) within each of the two groups. n = 3 technical replicates.